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## The Respiratory Burst of Phagocytes

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Studies on the oxygen metabolism of phagocytes began in 1933 with the observation that during phagocytosis, white blood cells (WBC) undergo a striking increase in oxygen consumption.<sup>1,2</sup> These observations did not attract much attention until the 1950s, when the metabolic activities of neutrophils during phagocytosis of foreign matter were reinvestigated.<sup>3-7</sup> The key initial observations that led to further research in the field were that (1) stimulated oxygen consumption is accompanied by enhanced catabolism of glucose through the hexose monophosphate shunt,<sup>4,6</sup> (2) inhibitors of mitochondrial oxidative respiration do not block oxygen consumption,<sup>5,6</sup> and (3) hydrogen peroxide is produced in parallel.<sup>8,9</sup>

During the past 30 years, the oxygen metabolism of phagocytes has been investigated in detail. Table I presents a provisional outline of the main observations of particular relevance in directing further research. Several important aspects are still unknown. However, some relevant conclusions have been reached on the significance of this peculiar cell function in the whole organism. These can be summarized as follows: (1) oxygen metabolism of the phagocyte represents an important mechanism of natural defense against invasion of microorganisms; (2) eukaryotic cells, including tumor cells, are also killed by products derived from the metabolism of oxygen; (3) abnormal or unrestrained stimulation of this function in phagocytes is responsible for the tissue damage associated with some important human diseases; and (4) *in vivo* modulation of phagocytes oxygen metabolism during the development of immunity mediated by cells of the T lineage is important in the acquired resistance against intracellular pathogens and tumor cell growth.

This chapter reviews some of main aspects of the respiratory burst of phagocytes at the cellular and the biochemical level. The interested reader can obtain a comprehensive view of this function in other detailed reviews that have appeared during the past few years.<sup>38-43</sup>

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TABLE I. Milestones in the Understanding of the Respiratory Burst of Phagocytes

Year(s)	Milestone	Reference
1933	Discovery that leukocytes enhance their oxygen consumption during phagocytosis	(1,2)
1956–1961	Start of the reinvestigation of the metabolism of oxygen in leukocytes and its definition as insensitive to mitochondrial inhibitors	(3,5–8)
1957–1959	Description of the activation of the hexose monophosphate shunt during phagocytosis	(4,6)
1961–1968	Identification of $H_2O_2$ as a product of the respiratory burst	(8,9)
1967–1970	Description of the $H_2O_2$ -myeloperoxidase-halide system as a powerful microbicidal system	(10–12)
1963–1964	Characterization of NADH and NADPH oxidases as the enzymatic basis of the respiratory burst	(13–16)
1973	Demonstration that superoxide anion is produced by stimulated neutrophils	(17)
1959–1975	Description of the chronic granulomatous disease as a defect of the respiratory burst and subsequently of the NADPH oxidase activity	(18–21)
1979	Demonstration that the NADPH oxidase is located in the plasma membrane	(22)
1971–1980	Demonstration that macrophages can be activated to produce higher amounts of toxic oxygen products	(23–27)
1966–1978	This is correlated with microbicidal and cytotoxic activities	(28–29)
	Discovery and subsequent reappraisal of cytochrome <i>b</i> as a unique phagocyte chromophore	(30–31)
	Discovery of its absence in CGD patients	(32,33)
1979	First attempts to solubilize and purify the NADPH oxidase	(34,35)
1984	Activation of the NADPH oxidase in a cell-free system by arachidonic acid	(36,37)

## 1. FEATURES OF THE RESPIRATORY BURST OF PHAGOCYTES

The pivotal studies on the events following phagocytosis cited in Table I led to the concept of respiratory burst to define the stimulation of the oxygen metabolism of phagocytes. This term is now used to describe a series of interconnected events, which include an increase in a nonmitochondrial oxygen consumption; the production of highly reactive oxygen species such as superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and other oxygen radicals; and the oxidation of glucose through the hexose monophosphate shunt.

This form of respiratory burst can be evoked in different types of phagocytic cells, i.e., in polymorphonuclear neutrophils and eosinophils, in circulating monocytes, and in tissue macrophages. Attempts to demonstrate the existence of a respiratory burst in other immune or inflammatory cells, e.g., cytotoxic T lymphocytes, natural killer (NK) cells, and platelets, produced controversial results.<sup>44–46</sup>

The respiratory burst is triggered by a wide and heterogeneous group of molecules capable of interacting with the phagocyte surface. Early studies showed that not only is phagocytosis able to stimulate oxygen consumption but that soluble agents have this capability as well.<sup>47–50</sup> The respiratory burst can be triggered by (1) stimuli that interact with specific surface receptors, e.g., chemotactic peptides,

immunoglobulin G (IgG), complement factors, leukotriene; (2) aggregation of surface molecules with antibodies and lectins; (3) degradation of plasma membrane phospholipids with exogenous phospholipase C; (4) unsaturated fatty acids and detergents; (5) elevation of cytosolic free calcium by ionophores; (6) stimulation of protein kinase C by phorbol diesters and diacylglycerol analogues; and (7) dissociation of G-protein subunits by fluoride. This simple list of stimuli and their different ways of interacting with the phagocyte gives an idea of the complexity of the mechanism(s) of activation of the respiratory burst. This point is addressed in some detail later on.

The respiratory burst is an impressive phenomenon with regard to the rapidity of its occurrence and intensity. Activation of the oxygen metabolism can be observed after a few seconds from the addition of an appropriate stimulus. In 1 min, one million stimulated cells can consume up to 5–10 nmoles of oxygen, something like 50–100 times more than resting cells. The intensity of the respiratory burst varies, depending on the cell type, the cell sources, and the animal species. Tissue macrophages are highly heterogeneous in their response and can be modulated by bacterial and cell-derived factors to metabolize oxygen in response to appropriate stimuli with a severalfold higher intensity.<sup>51–53</sup> The time course of the respiratory burst varies according to the stimulant used. For example, phagocytosable particles induce a progressive increase of respiration that accompanies the engulfing act and ceases when phagocytosis is completed,<sup>15</sup> chemotactic peptides trigger an almost instantaneous response that lasts only a few minutes, and phorbol diesters cause progressive irreversible activation that lasts several minutes in neutrophils and even 1 hr in macrophages. Stimulation of the respiratory burst can be reversed by removing the stimulus after washing the cells<sup>54</sup> or by adding an excess of a competitor for the same receptor.<sup>55</sup> Also, oxidative inactivation of the stimulus has been described.<sup>56</sup> Once deactivated, the respiratory burst can be reactivated by a second stimulus,<sup>56,57</sup> but desensitization of the system may occur. Both selective, i.e., due to receptor downregulation or uncoupling<sup>58–60</sup> and nonselective desensitization have been reported.<sup>59–62</sup>

TABLE II. Methods of Assaying the Respiratory Burst of Phagocytes

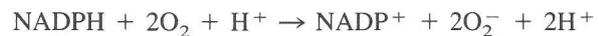
Method	Description
Oxygen consumption	Clark-type oxygen electrode
Hydrogen peroxide production	Fluorimetric measurement of peroxidase-catalyzed oxidation of scopoletin or of homovanillic acid; photometric detection of peroxidase spectrum; electron microscopic detection of cerium deposits
Superoxide anion production	Spectrophotometric measurement of cytochrome <i>c</i> reduction; microscopic examination of formazan formation in cells incubated in the presence of nitroblue of tetrazolium (NBT test)
Light production	Chemiluminescence detection with luminometer
Hexose monophosphate pathway activity	Production of <sup>14</sup> CO <sub>2</sub> from [1- <sup>14</sup> C]glucose

The set of phenomena included in the concept of respiratory burst can be studied by different well-established techniques based essentially on the measure of the total oxygen consumed, of the products of the oxygen reduction, and of the amount of glucose oxidized through the hexose monophosphate shunt. Table II lists the most frequently used methods in the study of the respiratory burst of phagocytes.

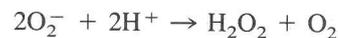
## 2. ENZYMATIC BASIS OF THE RESPIRATORY BURST

### 2.1. Major Properties of NADPH Oxidase, the $O_2^-$ -Generating System of Phagocytes

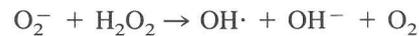
Studies on the enzymatic basis of the respiratory burst began during the 1960s in the laboratories of Karnovsky, Quastel, and Rossi. The matter soon attracted the attention of several groups of investigators and still remains the most challenging problem of the cell biology of phagocytes. It is now generally agreed that, as clearly proposed by Rossi and Zatti<sup>15</sup> initially, the enzymatic activity that accounts for the respiratory burst is a NADPH oxidase. This oxidase is apparently the only system present in eukaryotic cells able to perform exclusively a monovalent reduction of oxygen according to the reaction



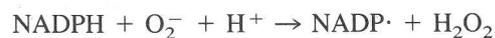
The  $O_2^-$  formed in this reaction can then give rise to other products known as reactive intermediates of oxygen reduction or toxic oxygen products such as hydrogen peroxide and hydroxyl radical. Hydrogen peroxide can derive from the spontaneous or the superoxide dismutase-catalyzed dismutation of  $O_2^-$ :



The formation of hydroxyl radical can derive from a secondary Haber-Weiss reaction, in which  $O_2^-$  reduces  $H_2O_2$ , probably with the obligatory catalysis of  $Fe^{2+}$  chelates:

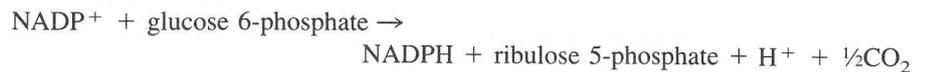


Other nonenzymatic chain reactions have been reported to occur at acidic pH and high NADPH concentrations and with the catalysis of  $Mn^{2+}$  ions<sup>63-65</sup>:



Among the set of enzymatic reactions set in motion by the NADPH oxidase, it is logical to include those linked to the hexose monophosphate shunt as well. Oxidation of NADPH to  $NADP^+$  decreases by threefold the ratio between the reduced and the oxidized form of pyridine nucleotides in phagocytosing neu-

trophils,<sup>66</sup> thereby activating the glucose 6-phosphate dehydrogenase (G6PD) to catalyze the reaction



Other pathways of activation of the hexose monophosphate shunt are those linked to the degradation (through the glutathione cycle) of the  $\text{H}_2\text{O}_2$  formed that enters the cytoplasmic space:



The main properties of the NADPH oxidase as an enzymatic system can be summarized as follows:

1. It is undetectable in resting cells and becomes active after exposure of the cells to appropriate stimuli. After stimulation for a few minutes at 37°C, cooling of the cells, and washing off of the stimulus, the activity can be easily assayed in disrupted cells. Subcellular fractionation studies have demonstrated that the activity copurifies with the plasma membrane and is apparently enriched in the phagosomal membrane.<sup>22,67</sup>
2. The optimum pH for the activity is 7.0–7.5.
3. Apparent  $K_m$  values are 0.015–0.08 for NADPH and 0.01–0.03 mM for  $\text{O}_2$ .
4. The activity is optimal in the presence of FAD, phospholipids, and low (0.02–0.05%) concentrations of detergents, if assayed in membrane preparations.
5. The activity is insensitive to inhibitors of oxidative phosphorylation ( $\text{CN}^-$ , antimycin A, rotenone) and inhibited by sulfhydryl-reactive agents (*p*-chloromercuribenzoate, mersalyl, *N*-ethyl maleimide), FAD analogues (5-carba-5-deaza FAD), pyridine nucleotide analogues (cibacron blue), metal chelating agents (bathophenanthroline sulfonate), and relatively high concentrations of detergents.
6. Appropriate concentrations of arachidonic acid<sup>36</sup> and sodium dodecyl sulfate (SDS)<sup>68</sup> can activate the superoxide-forming system in disrupted cells or in a reconstituted mixture composed of membranes or specific granules<sup>69</sup> plus cytosol.

## 2.2. Activity of the NADPH Oxidase in Relationship to the Respiratory Burst of Intact Phagocytes

The problem of the adequacy of the NADPH oxidase to account for the respiratory burst of intact phagocytes has been addressed by different investigators. Evidence has been provided that the activity of the oxidase in membrane fractions is

directly proportional to the respiration of intact cells. We have indeed shown that both the NADPH-dependent  $O_2^-$ -generating system and the NADPH-generating system, i.e., NADPH produced by the activity of the hexose monophosphate shunt, are competent to support the respiratory burst of intact phagocytes.<sup>70</sup>

A relevant aspect of the activity of the NADPH oxidase in relationship to the respiratory burst of the intact cell has recently emerged from studies on the molecular basis of the phenomenon of macrophage activation. Bacterial or cell-derived factors are well known to upmodulate the respiratory burst of tissue macrophages in response to appropriate stimuli; this phenomenon is directly linked to the acquisition of resistance against intracellular pathogens.<sup>51-53</sup> In macrophages activated by *in vivo* injection of bacteria, bacterial derivatives or other agents the NADPH oxidase was shown to display alteration in its kinetic properties with a striking 5- to 10-fold decrease in its  $K_m$ .<sup>71-73</sup> Significantly, a similar alteration was observed with human macrophages activated *in vitro* with  $\gamma$ -interferon (IFN $_{\gamma}$ ),<sup>74</sup> now considered the major macrophage activating factor derived from T lymphocytes. Furthermore, the inhibition of macrophage respiratory burst by factor(s) released by tumor cells is accompanied by an enhancement of the  $K_m$  of the NADPH oxidase.<sup>75</sup> These results substantiate the notion that the intrinsic capability of phagocytes to metabolize oxygen in response to environmental stimuli can be modulated by modifications of the affinity of the NADPH oxidase for its substrate.

### 3. NATURE OF NADPH OXIDASE

#### 3.1. An $O_2^-$ -Forming Electron-Transfer Chain

Although the properties of the NADPH oxidase as an enzymatic system capable of reducing the oxygen molecule monovalently are relatively well defined, its molecular structure is far from being clarified.

The bulk of evidence accumulated so far indicates that the  $O_2^-$ -generating system of phagocytes is formed by various components arranged as an electron-transport chain. The available data favor the following model of transport of reducing equivalents from NADPH to oxygen:



The participation of a flavoprotein as an NADPH-oxidizing component is suggested by various elements:

1. A flavoprotein is required for theoretical reasons because, whatever the electron acceptor (i.e., a cytochrome *b* or the oxygen itself), an intermediate electron carrier capable of transferring electrons from a two-electron donor (e.g., NADPH) to a one-electron acceptor would be obligatory.
2. A flavin cofactor in the form of FAD (but not other cofactors, e.g., FMN, riboflavin, ADP, AMP) was shown to be required to optimize NADPH oxidase activity of preparations solubilized with detergents.<sup>76</sup>

3. FAD analogues, e.g., 5-carba-5-deaza-FAD, inhibit the NADPH oxidase activity at micromolar concentrations.<sup>77</sup>
4. The addition of NADPH to preparations of solubilized oxidase in anaerobic conditions causes a reduction in FAD that is selective<sup>78</sup> or accompanied by cytochrome *b* reduction,<sup>79</sup> whether flavoproteins and cytochrome *b* are resolved by purification procedures or not, respectively.
5. Electron-spin resonance (ESR) studies showed a NADPH-dependent formation of flavin free radicals with characteristics of neutral semiquinone (FADH $\cdot$ ) in membrane preparations obtained from stimulated neutrophils.<sup>80</sup>

Studies during the past 10 years have found a *b*-like cytochrome to be the most substantial component of the O $_2^-$ -forming system of phagocytes. This hemoprotein was discovered during the 1960s and later defined as the major chromophore of neutrophils and other types of phagocytes. It was shown to have a peculiar peak of absorbance in the reduced state at 558 nm and a low midpoint potential of -245 mV.<sup>81</sup> Several lines of evidence suggest that cytochrome *b*<sub>558</sub> is a primary component of the O $_2^-$ -forming system:

1. Cytochrome *b*<sub>558</sub> is the major chromophore of phagocytes, including macrophages that contain mitochondrial and endoplasmic reticulum *b* cytochromes<sup>92</sup>; and its low midpoint potential is suitable for its role as an electron donor to oxygen, since the midpoint potential of the couple O $_2$ /O $_2^-$  is -330 mV.
2. During myelocyte differentiation, cytochrome *b* is expressed in parallel with the acquisition of the ability to undergo a respiratory burst.<sup>83</sup>
3. When neutrophils<sup>84</sup> or macrophages<sup>82</sup> are stimulated in an anaerobic environment, the cytochrome *b* is reduced and can then be rapidly reoxidized upon readdition of oxygen.
4. Cytochrome *b* behaves as a typical component of a terminal oxidase, as demonstrated by its ability to bind carbon monoxide<sup>85</sup>; it must be pointed out, however, that this finding was not confirmed by others.<sup>86</sup>
5. In anerobiosis, the addition of NADPH to membrane preparations or solubilized NADPH oxidase obtained from stimulated neutrophils is accompanied by a reduction in cytochrome *b*.<sup>79</sup>

Any hypothesis on the nature of the O $_2^-$ -forming activity of phagocytes must be compatible with the observations made on the cells of patients affected by chronic granulomatous disease (CGD). This genetic disease includes several variants transmitted either with an X-linked or autosomal-recessive mechanism. The common trait of all the described CGD syndromes is that in the homozygous form, the phagocytes are unable to undergo a respiratory burst in response to appropriate stimuli.

Phagocytes of CGD patients have been studied extensively to challenge the model of electron transfer from NADPH to oxygen described above. Several observations would indeed substantiate that model:

1. Phagocytes of patients affected by the X-linked form of CGD (X-CGD) are cytochrome *b* negative<sup>32,33</sup>; in the autosomal-recessive variant of the disease, stimulation of the cell in anaerobiosis is not accompanied by reduction of cytochrome *b*.<sup>87</sup>
2. A deficiency of flavoproteins or of FAD has been reported to occur in some CGD variants.<sup>88,89</sup>
3. Hybridization of monocytes of cytochrome *b*<sub>558</sub>-negative (e.g., *b*<sub>-558</sub>) CGD patients with monocytes of patients with a variant cytochrome *b*<sub>558</sub>-positive form (e.g., *b*<sub>558</sub>) reconstitutes the O<sub>2</sub><sup>-</sup>-forming activity, suggesting that the assembly of two or more components are required for this activity.<sup>90</sup>

Experimental observations supporting the model in which both a flavoprotein and the cytochrome *b*<sub>558</sub> are essential components of the O<sub>2</sub><sup>-</sup>-generating system are contradictory about the ratio between the two components. Either ratios of one flavoprotein for one cytochrome *b*<sup>88</sup> or one flavoprotein for 5–20 cytochrome *b*<sup>91,92</sup> have been reported (see Rossi *et al.*<sup>93</sup> for further discussion).

Although supported by most investigators in the field, there are grounds to conceive of models of electron transfer to oxygen that are different from that described. First, the participation of quinones<sup>94,95</sup> mediating electron transfer from a flavoprotein to cytochrome *b* can not be excluded. Claims have also been made that a flavoprotein<sup>96</sup> or an unidentified electron carrier<sup>97</sup> can transfer electrons directly to oxygen.

### 3.2. Molecular Structure of NADPH Oxidase

The structure of discrete components constituting the NADPH oxidase is still a matter of controversy. Attempts have been made in several laboratories to purify the oxidase but, up to now the results obtained are more contradictory than confirmatory of those of other investigators.

A major impediment to the purification of the oxidase is its extreme lability on detergent extraction and on manipulation by usual purification methods. Nonetheless, preparations of NADPH oxidase activity still conserving the O<sub>2</sub><sup>-</sup>-forming ability have been obtained in different laboratories by chromatographic techniques. The molecular weights of this preparation on gel-filtration chromatography ranged between 150,000 and 1,000,000. Electrophoresis of these preparations in polyacrylamide gels under denaturing conditions showed the presence of different bands with major components of 87,000<sup>98</sup> and 32,000.<sup>99,100</sup> With other methods of purification, active NADPH oxidase preparations gave, at electrophoretic analysis, components of 70,000,<sup>101</sup> 65,000,<sup>97</sup> or three major bands with molecular weights of 67,000, 48,000, and 32,000.<sup>96,102</sup>

To avoid some of the difficulties in purifying preparations conserving NADPH oxidase activity, different groups of investigators undertook the task of purifying to homogeneity the cytochrome *b*<sub>558</sub>. The rationale behind this approach is the possibility of detecting the spectrum of the reduced cytochrome *b* in preparations in

which the use of strong detergents has inactivated NADPH oxidase activity. Investigations performed at different laboratories have resulted in purification from human, pig, and bovine neutrophils of materials containing up to 20 nmoles cytochrome *b*/mg proteins, i.e., enriched about 200 times with respect to isolated membranes. Under nondenaturing conditions, this activity showed molecular weights of about 200,000. Electrophoretic analysis under denaturing conditions gave different molecular species. The reported molecular weights of major proteins present in partially purified preparations of cytochrome *b* are as follows: (1) 68,000–78,000  $M_r$  with conversion to 55,000  $M_r$  after carbohydrate cleavage<sup>103</sup>; (2) 11,000, 12,000, and 14,000  $M_r$ <sup>104</sup>; (3) 127,000  $M_r$ <sup>105</sup>; (4) 31,500  $M_r$  with minor species of 90,000–100,000  $M_r$ <sup>100</sup>; (5) 22,000 and 91,000  $M_r$  with conversion of this last to 48,000  $M_r$  after carbohydrate cleavage<sup>106</sup>; and (6) 23,000 and 78,000–90,000  $M_r$  species, which are absent in X-CGD.<sup>107</sup>

Recent results have provided extensive and definitive evidence that the cytochrome *b* of human neutrophils is a heterodimer composed of two polypeptides of 91,000–92,000 and 22,000–23,000  $M_r$  and that the former of this component is heavily glycosylated and can be converted to a lower molecular weight component of 50,000–55,000  $M_r$  after removal of N-linked sugars.<sup>108–110</sup>

The difficulties encountered to purify to homogeneity discrete components of the  $O_2^-$ -generating system are certainly one of the major reasons of the current uncertainty about the real nature of the NADPH oxidase. The most important new methodologies potentially able to overcome these difficulties, i.e. the isolation of monoclonal antibodies and gene-cloning techniques, do not appear to have been exploited at the level to push the field a step forward. Certainly, it should be only a question of time. Monoclonal antibodies against a partially purified oxidase preparation have been useful in identifying a novel proteolipid that is possibly involved in the mechanism of activation of the respiratory burst.<sup>111</sup> Elegant studies have permitted the cloning of a gene coding for the synthesis of a protein missing in one and abnormally transcribed in three patients with the X-CGD<sup>112</sup>; recently it has been shown that this is the high molecular weight component of the heterodimeric cytochrome *b* complex (see above).<sup>108,109</sup>

#### 4. MECHANISMS OF ACTIVATION OF THE RESPIRATORY BURST

The molecular events underlying the shift of the phagocytic cell from a quiescent state to that of a cell that actively metabolizes oxygen can be addressed in two different ways: (1) to define single discrete events that ensue from the perturbation of the plasma membrane with appropriate stimuli in order to establish a possible causal relationship between one of these and the eventual activation of the NADPH oxidase; and (2) to face the problem of the actual molecular modification of the NADPH oxidase, which is responsible for its conversion to a highly efficient system of electron transport.

Needless to say, the available information derives mainly from studies performed on the basis of the first way to address this central issue of the respiratory burst of phagocytes. In line with the burst of studies on mechanisms of transmembrane signaling in eukaryotic cells, the past few years have provided new insights into the molecular events that precede or accompany activation of the respiratory burst. These matters have been treated in detail in recent reviews.<sup>43,113</sup> What should be underscored is that the overwhelming evidence favors the view that specific receptors enable a GTP-binding protein to activate a phospholipase C that hydrolyzes, in a presumably selective way, phosphoinositides with formation of secondary messengers involved in elevating intracellular free calcium (inositol 3- and 4-phosphates) and in activating protein kinase C (diacylglycerol). Evidence has been presented, however, that activation of the respiratory burst can be completely dissociated from the phosphoinositide turnover<sup>114</sup>; therefore, it is not necessarily dependent on a unique and stereotyped sequence of events.

The molecular alteration(s) responsible for the conversion of the  $O_2^-$ -generating system to an active state are substantially conjectural and are based on indirect evidence. A comprehensive list of these alterations would include (1) a covalent, post-translational modification of NADPH oxidase components by phosphorylation, acylation, or methylation reactions; (2) a modification of the lipid environment in which the oxidase is embedded; and (3) the assembly of components of the electron-transfer chain, which can be either directly dependent on crosslinking or secondary to the above-mentioned modifications of proteins or lipids.

Evidence has been accumulated during the past few years that phosphorylation reactions are indirectly or directly linked to the activation of the NADPH oxidase. As originally shown by Schneider *et al.*,<sup>115</sup> activation of the respiratory burst of phagocytes is accompanied by the phosphorylation of several cytosolic and membrane proteins. The phosphorylation of three of these proteins shows a good correlation indeed with the activation of the respiratory burst in terms of dose dependence on the stimulus, kinetics, and effect of inhibitors.<sup>116,117</sup> Of these proteins, 64,000, 50,000, and 21,000  $M_r$ , only the last has been identified, probably the light chain of myosin. Also unknown is the role of a protein of 44,000 to 48,000  $M_r$ , which is phosphorylated in normal neutrophils but not in the neutrophils of patients with the autosomal-recessive form of CGD.<sup>118</sup> Since both the amount of cytochrome  $b_{558}$  and of flavoproteins in this CGD variant is normal, this protein is more likely involved in the activation mechanism rather than a component of the NADPH oxidase.

Ground for the role of phosphorylation reactions in the activation of the respiratory burst would also derive from the observation that the NADPH oxidase activity of neutrophils membranes can be stimulated by added purified brain protein kinase C in the presence of the appropriate cofactors necessary to sustain its activity.<sup>119</sup> However, to our knowledge, this results has not yet been confirmed by other investigators.

The only possible evidence of phosphorylation of a component of the NADPH oxidase was obtained in our laboratory and derives from the observation that the

32,000- $M_r$  protein that copurifies with the oxidase and constitutes a major species of highly purified preparations of cytochrome *b* is markedly phosphorylated upon cell stimulation.<sup>100,120</sup> Furthermore, in a cell-free system, phosphorylation of the 32,000- $M_r$  polypeptide was obtained in the presence of calcium, phosphatidylserine, and PMA, indicating that it is due to protein kinase C.<sup>121</sup>

In conclusion, it should be stated that the role of phosphorylation as an essential phenomenon in the conversion of the NADPH oxidase to its active state cannot be established until the oxidase components are defined more precisely. Until then, it can be cautiously stated that activation of the respiratory burst is accompanied by phosphorylation reactions for which the real significance on the response of the cell is unknown.

It has long been known that perturbation of the lipid environment of the phagocyte surface can activate the respiratory burst of phagocytes. Displacement of membrane cholesterol by saponin is an efficient stimulus of the respiratory burst.<sup>48,49</sup> Treatment of neutrophils with exogenous phospholipase C is also effective.<sup>50</sup> Finally, unsaturated fatty acids trigger the respiratory burst.<sup>122</sup> In the light of more recent data, the effect of phospholipase C can be explained with the production of diacylglycerol, the endogenous activator of protein kinase C; fatty acids have also been shown to trigger phosphorylation reactions.<sup>123</sup> However, the generation of secondary messengers by all the maneuvers that alter the lipid environment are far from conclusive. For example, the effect of saponin has not been reinvestigated, and the stimulatory activity of fatty acid is correlated with their capability to alter membrane fluidity.<sup>124</sup> Furthermore, one must face the intriguing evidence that arachidonic acid and even sodium dodecyl sulfate (SDS) are able to activate the NADPH oxidase in a cell-free system in conditions in which protein kinase C activity is not favored.<sup>36-37,68,125</sup> It is not yet possible to conclude whether activation of phospholipases by different types of stimuli converge in altering the lipid environment in which the NADPH oxidase is embedded and this causes its conversion to an active state. The perturbation of membrane lipids could indeed cause conformational modification of the oxidase which mimicks some more subtle modification operative in the response of the cell to physiologic environmental stimuli.

Independent of the mechanisms with which this would occur, there are some indications that the electron-transport chain could be assembled at the moment of stimulation. Early studies showed that nontoxic doses of glutaraldehyde inhibited the activation of the respiratory burst only if added before the stimulus, but not afterward.<sup>126</sup> More recently, another bifunctional crosslinking agent, *N*-hydroxy-succinimide, has been shown to have the same effect.<sup>127</sup> Significantly, cleavage of the crosslinker restores the neutrophil's response, indicating that aggregation of surface molecules plays indeed a role in stimulation of the respiratory burst. However, it is unknown whether the transmembrane signaling or the assembly of oxidase components is affected by the inhibition of the lateral diffusion of surface molecules. Evidence has also been presented that part of cytochrome  $b_{558}$  translocates from granules to the plasma membrane during activation of the respiratory burst<sup>128</sup> and appears to be incorporated in the phagocytic vacuoles membrane.<sup>129</sup>

Contradictory results have been obtained, however, with studies of localization<sup>130</sup> and also with the behavior of cytoplasts, which are devoid of granules but normally activatable.<sup>131</sup>

We think it reasonable to conclude that the plasma membrane pool of cytochrome *b* is sufficient to trigger the respiratory burst and that translocation of the intracellular pool is a concomitant event that might increase the efficiency or the duration of the oxidase activity. No direct evidence exists to show that assembly of the NADPH oxidase components are a necessary step in the activation of the respiratory burst.

## 5. BIOLOGICAL SIGNIFICANCE OF THE RESPIRATORY BURST OF PHAGOCYTES

Products derived from the activation of the phagocyte respiratory burst are now universally known as important mediators of host reactions. The most extensive knowledge on their role has been obtained by means of studies on the mechanism of killing of different pathogens. Products derived from the reduction of oxygen by the NADPH oxidase are part of a powerful microbicidal system,<sup>39</sup> and the ability of macrophages to kill intracellular pathogens is correlated with their oxygen metabolism.<sup>132</sup> Significantly, this last capability has been shown to be finely modulated during the development of cell-mediated immunity. IFN<sub>γ</sub> produced by activated T lymphocytes activates macrophage oxygen metabolism<sup>133</sup> and microbial killing in parallel.<sup>134</sup> More recently, it has been shown that members of the cytokine family, like granulocyte-macrophage colony-stimulating factor and IFN<sub>γ</sub> itself, are able to enhance the respiratory burst to environmental stimuli in neutrophils as well.<sup>135-138</sup> The modulation of phagocyte respiratory burst by cytokines produced by DNA technologies will certainly be exploited as a means of enhancing host resistance toward pathogens.

Several observations indicate that products of the respiratory burst can also have the ability, by themselves or in concert with other factors, to damage eukaryotic cells. The hydrogen peroxide-halide peroxidase system has long been recognized as a mediator of cytotoxicity.<sup>139,140</sup> Good correlation has been found between the ability of neutrophils and different populations of macrophages to undergo a respiratory burst and to lyse tumor cells in parallel.<sup>29</sup> Finally, the action of both neutrophils and macrophages as effector cells in antibody-dependent cell-mediated cytotoxicity has been shown to be mediated as well by molecules produced during activation of the respiratory burst.<sup>141,142</sup> These data encourage studies on the effect of *in vivo* modulation of phagocytes respiratory burst by IFN<sub>γ</sub> and other cytokines in controlling tumor cell growth.

Cell damage consequent to activation of phagocytes respiratory burst can conceivably accompany inflammatory processes where such complement factors as C5a, leukotriene, platelet-activating factor, or immobilized immune complexes are

present. In the clinical situation known as adult respiratory distress syndrome (ARDS) or shock lung, C5a generated during passage of blood through membranes used for extracorporeal haemodialysis, or during burns, trauma, endotoxin intoxication causes aggregation of neutrophils; the activation of the respiratory burst that ensues is responsible for damage to endothelial cells, followed by extravasation of blood constituents.<sup>143,144</sup>

Products of the respiratory burst can have even more far-reaching effects on the development and regulation of the inflammatory process than that caused by induction of cell injury. Evidence has in fact, been presented to show that these products can affect other cell functions, such as platelet aggregation and secretion, endothelial cell permeability, mast cell secretion. Fibrous proteins, hyaluronic acid, and proteoglycans of connective tissues, enzymes, and protein inhibitors can also be targets of oxygen metabolites undergoing structural and functional alteration.

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